



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>A61K 037/02, 045/05</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 91/07992</b> <b>(43) International Publication Date:</b> 13 June 1991 (13.06.91)
<b>(21) International Application Number:</b> PCT/AU90/00556 <b>(22) International Filing Date:</b> 20 November 1990 (20.11.90) <b>(30) Priority data:</b> PJ 7552 24 November 1989 (24.11.89) AU <b>(71) Applicant (for all designated States except US):</b> MONASH UNIVERSITY [AU/AU]; Wellington Road, Clayton, VIC 3168 (AU). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> AUSTIN, Lawrence [AU/AU]; 9 Madison Court, Mount Waverley, VIC 3149 (AU). <b>(74) Agents:</b> SLATTERY, John, M. et al.; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).		<b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.  <b>Published</b> <i>With international search report.</i>

**(54) Title:** PROLIFERATIVE ACTION OF LEUKAEMIA INHIBITORY FACTOR ON SATELLITE CELLS**(57) Abstract**

The present invention relates to the use of leukaemia inhibitory factor (LIF), alone or in combination with other cytokines such as interleukin-6 (IL-6) and/or transforming growth factor  $\alpha$  (TGF $\alpha$ ) and/or fibroblast growth factor (FGF), to stimulate the proliferation and/or differentiation of mammalian satellite cells. The present invention also contemplates a method comprising myoblast transfer therapy whereby LIF, alone or in combination with other cytokines, is/are employed to proliferate and/or differentiate mammalian satellite cells into myoblasts. The present invention is also directed to a cell activating composition and a pharmaceutical composition comprising LIF alone or in combination with other cytokines to promote proliferation and/or differentiation of mammalian satellite cells *in vitro* and *in vivo*, respectively.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CI	Côte d'Ivoire	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

**PROLIFERATIVE ACTION OF LEUKAEMIA INHIBITORY  
FACTOR ON SATELLITE CELLS**

5

The present invention relates to the use of leukaemia inhibitory factor (LIF), alone or in combination with other cytokines such as interleukin-6 (IL-6) and/or transforming growth factor  $\alpha$  (TGFA) and/or fibroblast growth factor (FGF), to stimulate the proliferation and/or differentiation of mammalian satellite cells. The present invention also contemplates a method comprising myoblast transfer therapy whereby LIF, alone or in combination with other cytokines, is/are employed to proliferate and/or differentiate mammalian satellite cells into myoblasts. The present invention is also directed to a cell activating composition and a pharmaceutical composition comprising LIF alone or in combination with other cytokines to promote proliferation and/or differentiation of mammalian satellite cells in vitro and in vivo, respectively.

Skeletal muscle consists of parallel arrays of multinucleated cells which are innervated and attached to bone through tendons. Although these highly differentiated cells are not capable of replication, muscle has a high capacity for regeneration after injury or disease and this is achieved by the activation of stem cells, called satellite cells, which lie in close association with muscle fibres. It has been estimated that up to 20% of muscle cell nuclei are found in satellite cells.

On activation, satellite cells differentiate into elongated mononuclear myoblasts. These, when in sufficient numbers, fuse to form multinucleated myotubes, the progenitor of the muscle fibre.

Primary cultures of muscle cells all originate from the satellite cells. The muscle is minced and treated with trypsin to break up fibres and extracellular matrix. Satellite cells, released as a result of this process,  
5 are harvested and placed under cell culture conditions.

After a lag period of about three days, the cells proliferate and undergo differentiation into myoblasts. These also proliferate and when the culture reaches  
10 confluence, the cells begin to fuse to form multinucleated myotubes. The cells may be passaged many times, but this must be done at the myoblast stage, before fusion.

15 The nature of the control of proliferation of satellite cells and subsequent differentiation into myoblasts is not well known although it has been discovered that the heparin binding growth factor, fibroblast growth factor (FGF), stimulates growth of satellite cells (Di Mario and  
20 Stohman, Differentiation 39:42-49, 1988).

The present invention arose in part from a study into the effect of a variety of cytokines on the early stages of muscle cell growth in culture. In accordance with the  
25 present invention, it has been discovered that LIF and to a lesser extent other cytokines such as IL-6 and TGF $\alpha$ , stimulate the proliferation of satellite cells and the subsequent development of myoblasts.

30 Accordingly, one aspect of the present invention relates to a method of stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts which method comprises contacting said cells with a stimulation-effective amount of LIF for a time and  
35 under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts.

Another aspect of the present invention relates to a method of stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts which method comprises contacting said cells with a stimulation-effective amount of LIF in simultaneous or sequential combination with one or more other cytokines, for a time and under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts.

10

Still another aspect of the present invention contemplates a method of myoblast transfer therapy comprising contacting mammalian satellite cells with a proliferation- and/or differentiation- effective amount of LIF for a time and under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts and then administering said myoblasts at multiple sites into muscles. In an alternative to this embodiment, LIF is used in simultaneous or sequential combination with one or more other cytokines.

20

Yet another aspect of the present invention relates to a cell activating composition comprising LIF in combination with one or more other cytokines, and one or more physiologically acceptable carriers and/or diluents.

25

Still yet another aspect of the present invention relates to the use of LIF, alone or in combination with one or more other cytokines, in the manufacture of a cell activating composition for stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts.

30

In still yet another aspect of the present invention there is provided a pharmaceutical composition for stimulating the proliferation and/or differentiation of satellite cells comprising LIF and one or more other

35

cytokines and one or more pharmaceutically acceptable carriers and/or diluents.

5 In one preferred embodiment, the cytokines in optional combination with LIF include IL-6 and/or TGF $\alpha$  and/or FGF.

10 The satellite cells and cytokines may originate from homologous or heterologous mammals. If the same mammal is used, satellite cells and cytokines may originate from the same or different species of the same mammal. Mammals contemplated herein include but are not limited to humans, mice and livestock animals.

15 The present invention extends to naturally occurring (native), recombinant and/or synthetic cytokines and/or to their derivatives and/or analogues and/or to any combinations thereof. For example, recombinant murine and human LIF are disclosed in International Patent Application No. PCT/AU88/00093. Use of the term "LIF"  
20 herein encompasses all such forms of LIF and its derivatives and analogues and includes single or multiple amino acid substitutions, deletions and/or additions to the polypeptide portion of the LIF molecule and single or multiple substitutions, deletions and/or additions to the  
25 carbohydrate portion of the molecule (when present). Derivatives and analogues of LIF include portions of native, recombinant and/or synthetic LIF which have the desired activity.

30 By "simultaneous or sequential combination" as used herein means the addition of LIF and one or more other cytokines at the same time, i.e. in a single composition or the administration of each active molecule or groups thereof one after the other. By way of non-limiting  
35 example, LIF may be used first followed by a second cytokine followed by a third cytokine and so on. Alternatively, LIF may be used first followed by a

combination of other cytokines. In another embodiment, other cytokines are used first (simultaneously or sequentially), followed by LIF.

5 The present invention is of medical significance especially in relation to primary, genetically determined, muscle myopathies. There are considerable numbers of these, the most severe and the most common of which, being Duchenne muscular dystrophy (DMD). The  
10 affected gene is known and its protein product has been deduced. The protein product, dystrophin, is probably a component of the cytoskeleton, membrane linkage. It is large, 425,000 daltons, and the gene is the largest of the human genes. Because of its complexity, it is  
15 unlikely that genetic manipulation will be possible in the near future. However, another approach has been shown to be effective in mouse models of muscular dystrophies, including the mdx mouse.

20 This approach involves the growing of myoblasts in culture derived from normal mice and injecting them, at multiple sites, into muscles of mutant mice. The results have shown that not only has rejection been minimal, but the muscles contain dystrophin whereas previously there  
25 was none.

In a mouse strain showing very severe muscle wastage (not dystrophin deficient, but a mutant with an unknown defect) muscle strength returned to near normal.

30 Thus, a procedure is contemplated termed myoblast transfer therapy, whereby human myoblasts, grown in culture, are injected at multiple sites into muscles of DMD. This approach is applicable to all primary  
35 myopathies and not only DMD.

The process involves injection of many myoblasts at multiple sites in a large number of muscles. It is time-consuming and the cost of myoblast culture is high. At present, techniques of culturing myoblasts utilize medium to long term culture with varying concentrations of the expensive reagent fetal calf serum (FCS). Thus, any factor which may accelerate the myoblast differentiation and growth should be significant in reducing the cost of myoblast production. In accordance with the present invention, therefore, LIF alone, or in combination with other cytokines such as IL-6 and/or TGF $\alpha$  and/or FGF, will fulfill this need.

Accordingly, one aspect of the present invention is directed to a method of stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts comprising contacting said satellite cells with a stimulation-effective amount of LIF, alone or in combination with other cytokines such as IL-6 and/or TGF $\alpha$  and/or FGF, for a time and under conditions sufficient to stimulate the satellite cells.

The present invention is also directed to cell activating compositions comprising LIF with or without other cytokines such as IL-6 and/or TGF $\alpha$  and/or FGF and one or more physiologically acceptable carriers and/or diluents. Preferably, the compositions comprise LIF in combination with one or more other cytokines.

The present invention is also directed to a pharmaceutical composition for stimulating the proliferation and/or differentiation of mammalian satellite cells which composition comprises LIF with one or more other cytokines and one or more pharmaceutically acceptable carriers and/or diluents. In one embodiment, the cytokines other than LIF contemplated herein include IL-6 and/or TGF $\alpha$  and/or FGF. Methods for preparing a



pharmaceutical composition are known in the art such as described in Remington's Pharmaceutical Sciences 16th ed., 1980, Mack Publishing Co., edited by Osol et al. and hereby incorporated by reference. The route of administration and the effective amount of active component will be determined by the situation but one preferred route is intramuscular although other routes of administration may be employed. For the purposes of exemplification of this embodiment of the present invention, the effective amount of LIF used is from about 0.1 to about 1000 U/ml, preferably about 1 to about 100 U/ml and most preferably from about 10 to about 50 U/ml. A unit of LIF is defined in PCT/AU88/00093. In general, other cytokines will be used at from about 1 to about 100 ng/ml. More specifically, IL-6 may be preferably used at a concentration of about 60 to about 100 ng/ml; TGF $\alpha$  preferably at about 1 to about 20 ng/ml; and FGF preferably at about 20 to about 50 ng/ml. These concentrations may vary depending on the circumstances and it is not the intention to necessarily limit the present invention to these effective amounts.

The present invention is further described by the following non-limiting Figures and Examples.

In the Figures:

Figure 1 is a graphical representation showing the response of passaged cells to LIF at several concentrations of FCS. Passaged mouse myoblasts (P2) were plated into 96 well plates at 2500 cells per well, in Ham F12 medium containing 5, 7.5 or 10% v/v FCS. LIF was added at the concentrations shown and the cell grown without further change of medium. Cell numbers were counted at the times shown and are expressed as cells/mm<sup>2</sup> of well surface.

Figure 2 is a graphical representation showing the effect of LIF on human myoblasts. Human myoblasts were derived from a sample of human skeletal muscle, grown to about 80% confluence and passaged. These passaged cells were grown in the presence of LIF, as described for Figure 1.

Figure 3 is a graphical representation showing the effect of TGF- $\alpha$  replaced LIF at the concentrations shown.

Figure 4 is a graphical representation showing the effect of FCS concentration of LIF activity. LIF and FCS human myoblasts were grown as described for Figure 2. FCS, ranging from 0 to 20% v/v was added to the Ham F12 medium before addition of LIF. (a) 0% - 5% v/v FCS, (b) 7.5% - 20% v/v FCS.

Figure 5 is a photographic representation showing fusion of LIF-supported myoblasts in culture. Human myoblasts were grown either in the absence or presence of 30 units/ml LIF. These were harvested, suspended in PBS and injected into one extensor digitorum longus of mdx mice. The mice were killed 5 weeks later and the muscle was embedded for cryostat sectioning. Sections were treated with anti-dystrophin antibodies and the presence of dystrophin was visualised using fluorescein labelled anti-sheep antibodies. (A) Muscle of C57-BL-10 normal, dystrophin positive mouse. (B) Muscle of non-injected mdx, dystrophin negative mouse. (C) Muscle from mdx mouse injected with myoblasts grown in the presence of LIF. Note patches of dystrophin - positive areas underlying the sarcolemma.

Figure 6 is a photographic representation showing LIF receptors on myoblasts. Autoradiography of mouse myoblasts subjected to  $^{125}\text{I}$ -LIF. (a)  $^{125}\text{I}$ -LIF alone. (b)  $^{125}\text{I}$ -LIF in the presence of a 1000-fold excess of non-labelled LIF.

**EXAMPLE 1**  
**MATERIALS AND METHODS**

Mouse muscle cells:

5 The muscle used was from the hind legs of mouse strain CS7/BL/10. A mutant from the same strain was also used. This is a mdx mutant in which the muscle protein dystrophin is missing. This is an excellent model to study muscular dystrophy since the same gene is affected  
10 in the human condition.

Primary cultures of these cells were grown as described by Gurusingha et al. Muscle and Nerve 11: 1231-1239, 1988, except that 5 - 10% v/v FCS was used. When at  
15 about 80% confluence, the cells were washed briefly with PBS and treated with 0.025% w/v trypsin in dissociation buffer to detach them. Foetal calf serum (FCS) was added to 5% v/v concentration to inhibit the trypsin, the cells centrifuged at 1000 rpm for 10 minutes and washed twice  
20 with PBS. They were then plated at 10% confluence in Ham F12, 20% v/v FCS to provide passaged cells.

Human muscle cells:

Under these conditions, many cells survive and  
25 differentiate, but at a rate lower than those under optimum conditions in which 20% (v/v) FCS is used. Usually there is a lag period of 3 - 4 days during which time cell numbers tend to decrease followed by the appearance of myoblasts at day 5 - 7. The cells are  
30 initially plated into 96 well cluster plates at a density of 3-5000 cells/well. Growth factors are added 3 days after initiation of the culture and the effect quantitated by counting cells as satellite cells or myoblasts over a period.

Samples (0.5 - 1.5g) of human skeletal muscle were removed by collaborating surgeons from consenting patients during operative procedures. Human Ethics Committee Approvals are held from Monash University, the Monash Medical Centre, Clayton and the Royal Children's Hospital, Parkville. These samples were transported back to the laboratory and cultures commenced, essentially as described for mouse cells.

10 In vivo myoblast transfer:

Passaged mouse or human myoblasts were grown to 80 - 90% confluence in the absence or presence of 30 units/ml LIF and harvested as described above. They were suspended in PBS at  $3 \times 10^5$  cells/ml. Mutant mdx mice, 25 - 32 days of age were anaesthetised by I.P. injection of a mixture of hypnorm (0.3 ml/kg) and diazepam (5mg/kg). One extensor digitorum longus (EDL) muscle was exposed and myoblasts were injected. These cells were delivered in 1 $\mu$ l lots from a SGE syringe fitted with a needle that had been electropolished at the tip to 27 gauge. The syringe was mounted on a micromanipulator to control position and depth of injection. Four or five injections were made into each muscle, at intervals of 1.5 - 2mm. Control injections were carried out using PBS alone. The wound was sutured and the mouse allowed to recover.

After 4 to 6 weeks, the animals were killed by breaking the neck, the EDL muscle re-exposed, cooled with ice cold PBS, removed and embedded in Tissue - Tek OCT and immediately quenched in isopentane at liquid N<sub>2</sub> temperature. The blocks were trimmed at -25° and transverse sections cut in a cryostat at -20° to 3 - 4  $\mu$ m. When air-dried, the sections were treated with either 60 Kd or 30 Kd anti-dystrophin antibodies (Hoffman et al. Cell 51: 919-928, 1987) diluted 1/200 with PBS. Pre-immune serum was similarly diluted. After incubation at R.T. for 30 minutes at 100% humidity, the sections were

washed three times and then subjected to FITC donkey -  
antisheep antibodies (Silenius, Melbourne) at 1/40  
dilution. They were again washed and mounted under  
coverslips.

5

## EXAMPLE 2

### Mouse Myoblasts:

Primary cultures of mouse myoblasts were passaged and  
10 grown in medium containing LIF at various concentrations.  
This procedure was carried out three times in medium  
containing 5%, 7.5% and 10% v/v FCS. Thus, it was  
possible to test whether passaged cells respond to LIF in  
the same manner as primary cultures and also to examine  
15 the response to LIF under various growth conditions.

Figure 1 shows that passaged mouse cells respond to LIF  
at several cell concentrations of FCS. The optimum  
concentration of LIF is 30 units/ml (14pg/ml) as it was  
20 for primary cultures. There was a greater effect of LIF  
at 10% v/v FCS concentration than at lower  
concentrations, this being a 13 fold increase over  
controls.

### 25 Human myoblasts:

These cells were grown from donor human muscle and  
passaged cells were seeded at 2-3000 cells per well in a  
96 well cluster plate in Ham F12 medium containing 7.5%  
v/v FCS as was carried out for initial mouse cell  
30 cultures. The medium contained varying concentrations of  
LIF. Cell numbers were counted at times up to 12 days  
and the results are shown in Figure 2. As with mouse  
cells, there was a marked stimulation of proliferation of  
myoblasts by LIF. Again the optimum concentration found  
35 for LIF was 30 units/ml. This shows that human myoblasts  
respond to LIF in a manner similar to mouse myoblasts.

It has previously been found that TGF- $\alpha$  also stimulates mouse myoblasts. Human cells were grown also in the presence of this cytokine at concentrations ranging up to 10 ng/ml. Figure 3 shows that there was early response to TGF- $\alpha$ , with an optimum concentration of 1 ng/ml, the same as that found for mouse cells. As was the case for LIF, higher concentrations were less effective than that at the optimum. Unlike mouse cells, the response occurred early. This may be due to species differences or passaging of cells or some other unknown factors.

Effects of FCS concentrations of LIF activity:

Passaged human myoblasts were grown in Ham F12 medium containing FCS concentrations ranging from 0-20% v/v FCS. At each of the FCS concentrations, LIF was added at 0, 30 or 100 units/ml. Figure 4 shows that at 0 and 1% v/v FCS there is no cell growth in the absence or presence of LIF. When the FCS concentration is 2% v/v, again there is no growth in the absence of LIF, but some growth occurs in its presence. At increasing concentrations of FCS, LIF provides increased growth and as shown previously, 30 units/ml of LIF is more effective than 100 units/ml, at all FCS levels. Optimum stimulation of growth by LIF was found to occur at 15% v/v FCS.

In vivo myoblast transfer:

Mice were injected with either mouse or human myoblasts cultured in the presence of LIF as described in Example 1 at the rate of  $1 - 1.5 \times 10^6$  cells per EDL muscle. These were killed and the muscles prepared for immunocytochemistry 4 - 6 weeks later. Figure 5A shows the presence of dystrophin in an EDL muscle of the C57-BL-10 dystrophin positive control mouse strain. As described by others (Partridge *et al.* Nature 337: 176-179, 1989) the dystrophin is located under the surface of the sarcolemma membrane. The mdx dystrophin negative muscle is shown in Figure 5B. No immunoreaction is evident.

Figure 5C shows EDL muscle sections from an mdx mouse injected 6 weeks earlier with human myoblasts. That fusion has occurred can be seen from dystrophin positive patches located at the sarcolemma of the fibres.

- 5 Positive fusion was also found when mouse myoblasts were injected into the EDL muscles.

LIF receptors on myoblasts:

- 10 Mouse myoblasts were grown on glass slides, pretreated with fibronectin to ensure good adhesion. After eight days in culture, they were subjected to <sup>125</sup>I-labelled LIF in the absence and presence of a 1000 fold excess of unlabelled LIF. Figure 6A shows that LIF receptors are present on myoblasts, whereas Figure 6B shows low non-specific binding.
- 15

- Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any
- 20
- 25 and all combinations of any two or more of said steps or features.

## CLAIMS:

1. A method of stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts which method comprises contacting said cells with a stimulation-effective amount of leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts.
2. The method according to claim 1 which further comprises the addition of one or more other cytokines in simultaneous or sequential combination with LIF.
3. The method according to claim 1 or 2 wherein the LIF, cytokine and/or satellite cells are from the same mammal.
4. The method according to claim 3 wherein the LIF cytokine and/or satellite cells are from the same species of mammal.
5. The method according to claim 1 or 2 wherein the LIF, cytokine and/or satellite cells are from different mammals.
6. The method according to any one of claims 1 to 5 wherein the mammal is human, mouse or a livestock animal.
7. The method according to claim 1 or 2 wherein the LIF and/or cytokine is prepared by recombinant or synthetic means.
8. The method according to any one of the preceding claims wherein the cytokine is one or more of IL-6, TGF $\alpha$  and/or FGF.



9. The method according to any one of the preceding claims wherein LIF is provided at a concentration of from about 0.1 to about 1000 U/ml and the cytokine is provided at a concentration of from about 1 to about 100 ng/ml.
10. The method according to claim 9 wherein LIF is provided at a concentration of from about 1 to 100 U/ml.
11. The method according to claim 10 wherein LIF is provided at a concentration of from about 10 to about 50 U/ml.
12. A method of myoblast transfer therapy comprising contacting mammalian satellite cells with a proliferation- and/or differentiation-effective amount of LIF for a time and under conditions sufficient for said satellite cells to proliferate and/or differentiate into myoblasts and then administering said myoblasts at multiple sites into muscles.
13. The method according to claim 12 which further comprises the addition of one or more other cytokines in simultaneous or sequential combination with LIF.
14. The method according to claim 12 or 13 wherein the LIF, cytokine and/or satellite cells are from the same mammal.
15. The method according to claim 14 wherein the LIF, cytokine and/or satellite cells are from the same species of mammal.
16. The method according to claim 12 or 13 wherein the LIF, cytokine and/or satellite cells are from different mammals.

17. The method according to any one of claims 12 to 16 wherein the mammal is human, mouse or a livestock animal.
18. The method according to claim 12 or 13 wherein the LIF and/or cytokine is prepared by recombinant or synthetic means.
19. The method according to any one of the preceding claims wherein the cytokine is one or more of IL-6, TGF $\alpha$  and/or FGF.
20. The method according to any one of claims 12 to 19 wherein LIF is provided in a concentration of from about 0.1 to about 1000 U/ml and the cytokine is provided at a concentration of from about 1 to about 100 ng/ml.
21. The method according to claim 20 wherein LIF is provided at a concentration of from about 1 to about 100 U/ml.
22. The method according to claim 21 wherein LIF is provided at a concentration of from about 10 to about 50 U/ml.
23. The method according to any one of claims 12 to 20 wherein the myoblasts so obtained are administered by intramuscular injection.
24. A cell activating composition comprising LIF in combination with one or more other cytokines and one or more physiologically acceptable carriers and/or diluents.
25. The composition according to claim 24 wherein the LIF, cytokine and/or satellite cells are from the same mammal.

26. The composition according to claim 25 wherein the LIF, cytokine and/or satellite cells are from the same species of mammal.
27. The composition according to claim 24 wherein the LIF, cytokine and/or satellite cells are from different mammals.
28. The composition according to any one of claims 24 to 27 wherein the mammal is human, mouse or a livestock animal.
29. The composition according to claim 24 wherein the LIF and/or cytokine is prepared by recombinant or synthetic means.
30. The composition according to any one of the preceding claims wherein the cytokine in combination with LIF is one or more of IL-6, TGF $\alpha$  and/or FGF.
31. The use of LIF in the manufacture of a cell activating composition for stimulating the proliferation and/or differentiation of mammalian satellite cells into myoblasts.
32. The use according to claim 31 further comprising the use of one or more other cytokines in simultaneous or sequential combination with LIF.
33. The use according to claim 31 or 32 wherein the mammal is human, mouse or livestock animal.

1/9

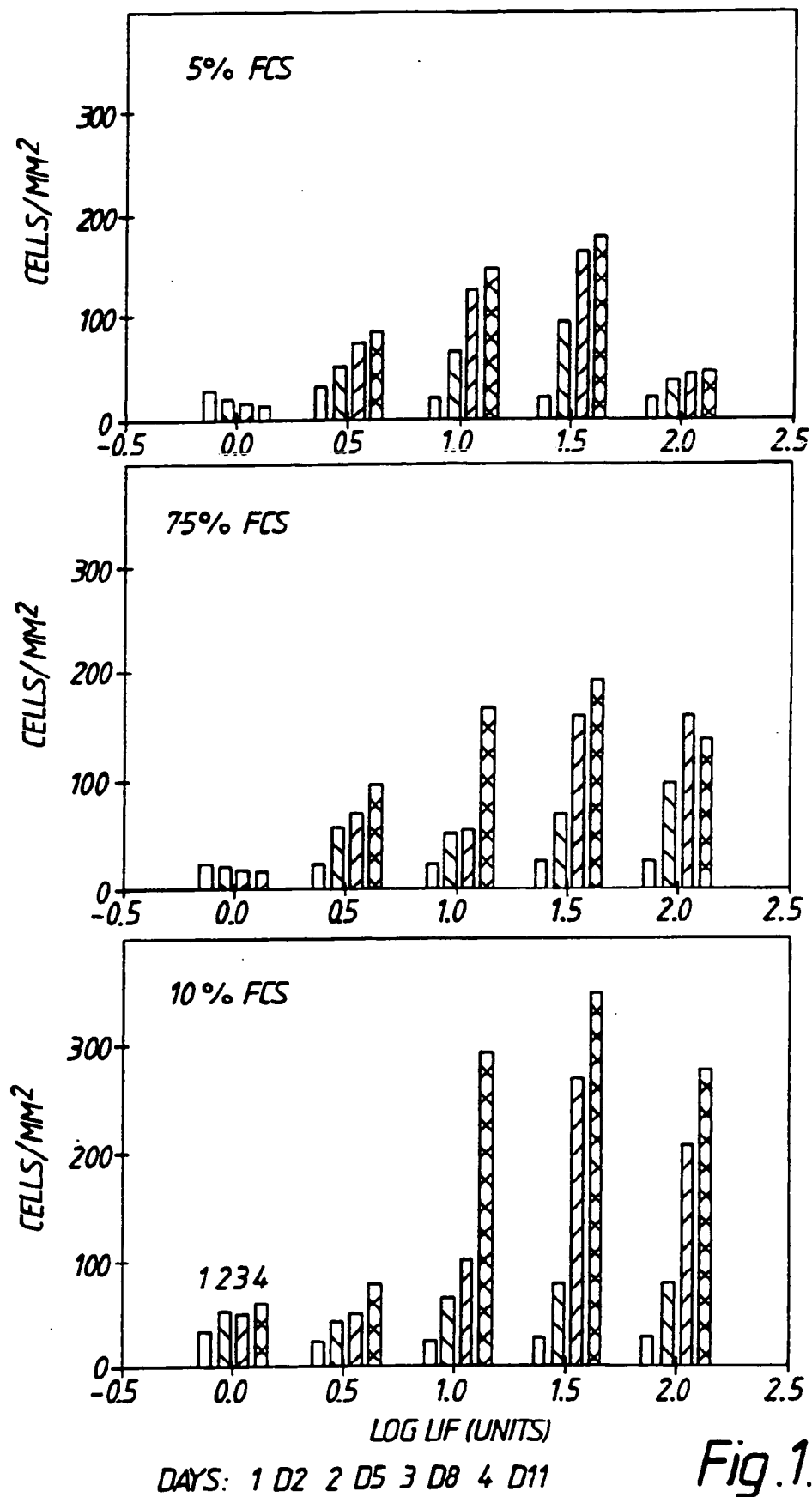


Fig.1.

2/9

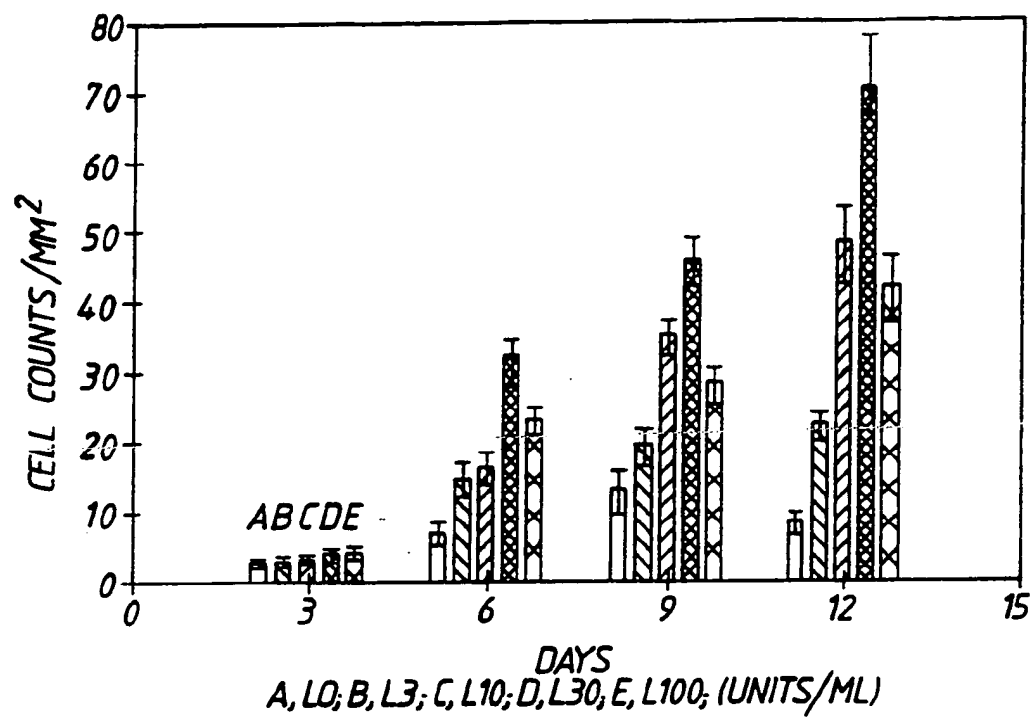


Fig.2.

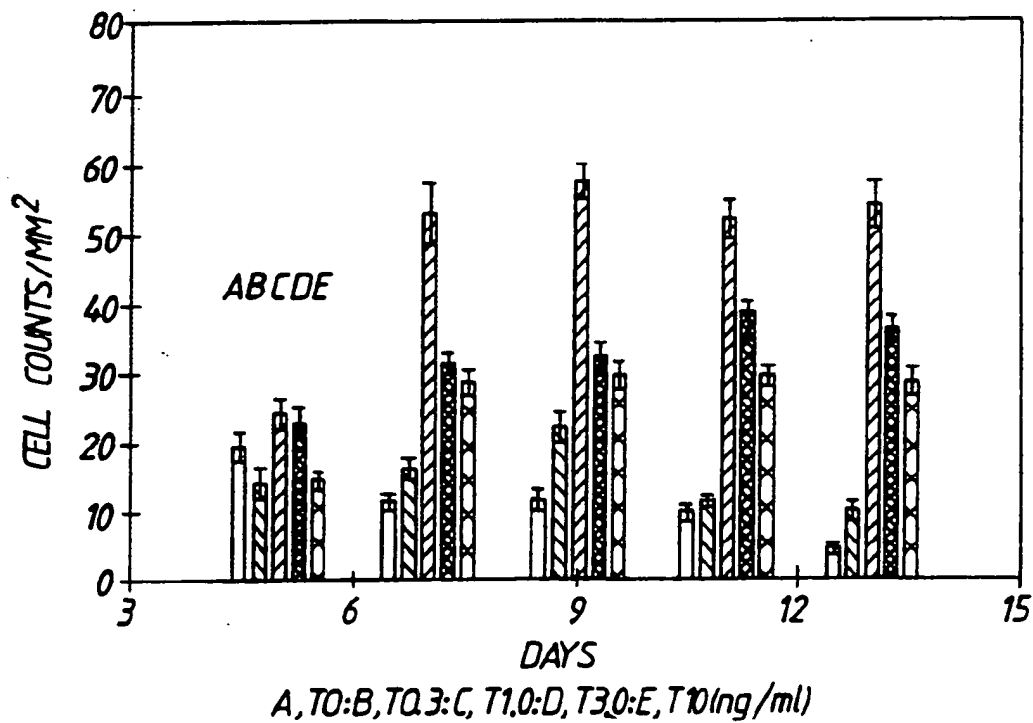


Fig.3.

3/9

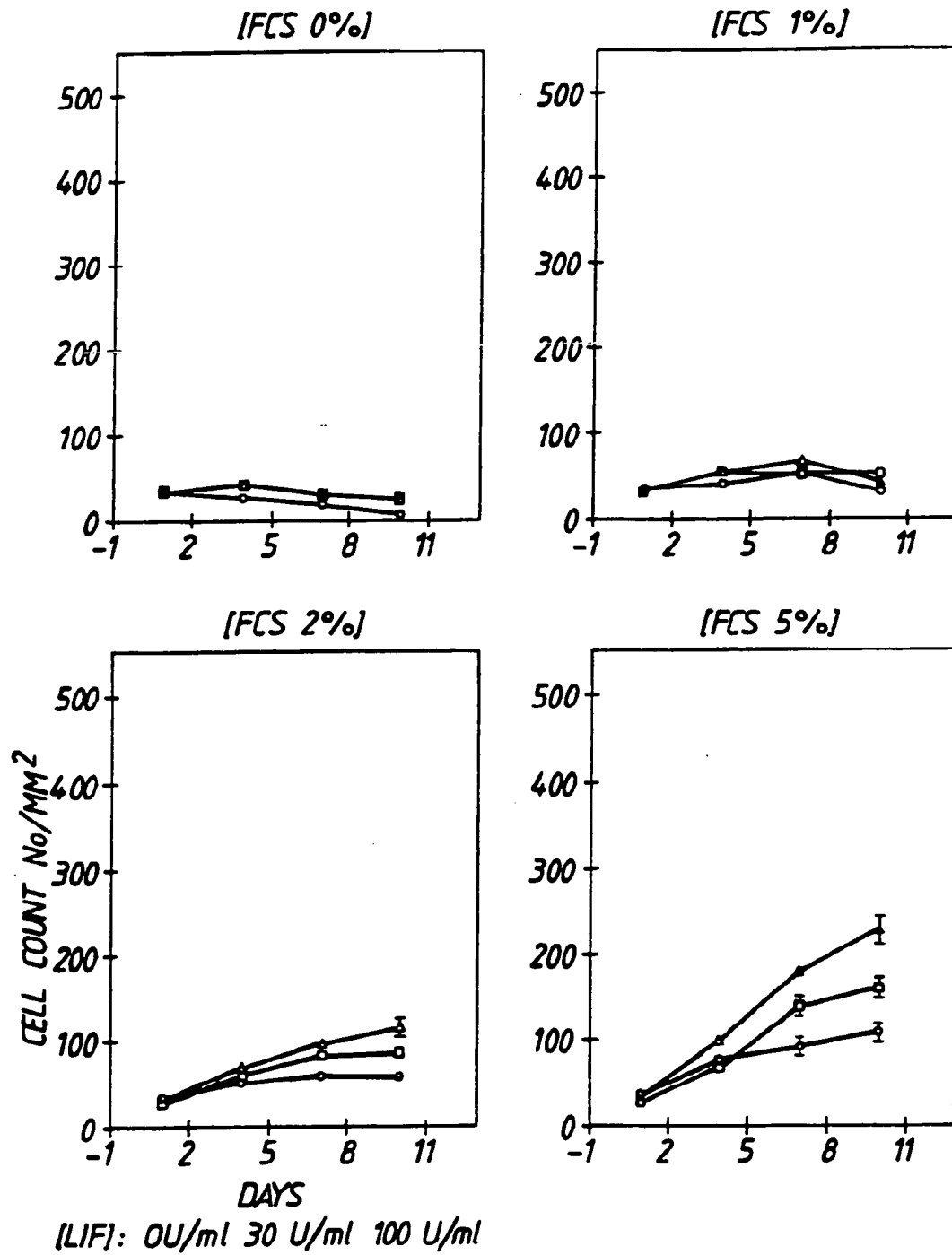


Fig. 4a.

SUBSTITUTE SHEET

4/9

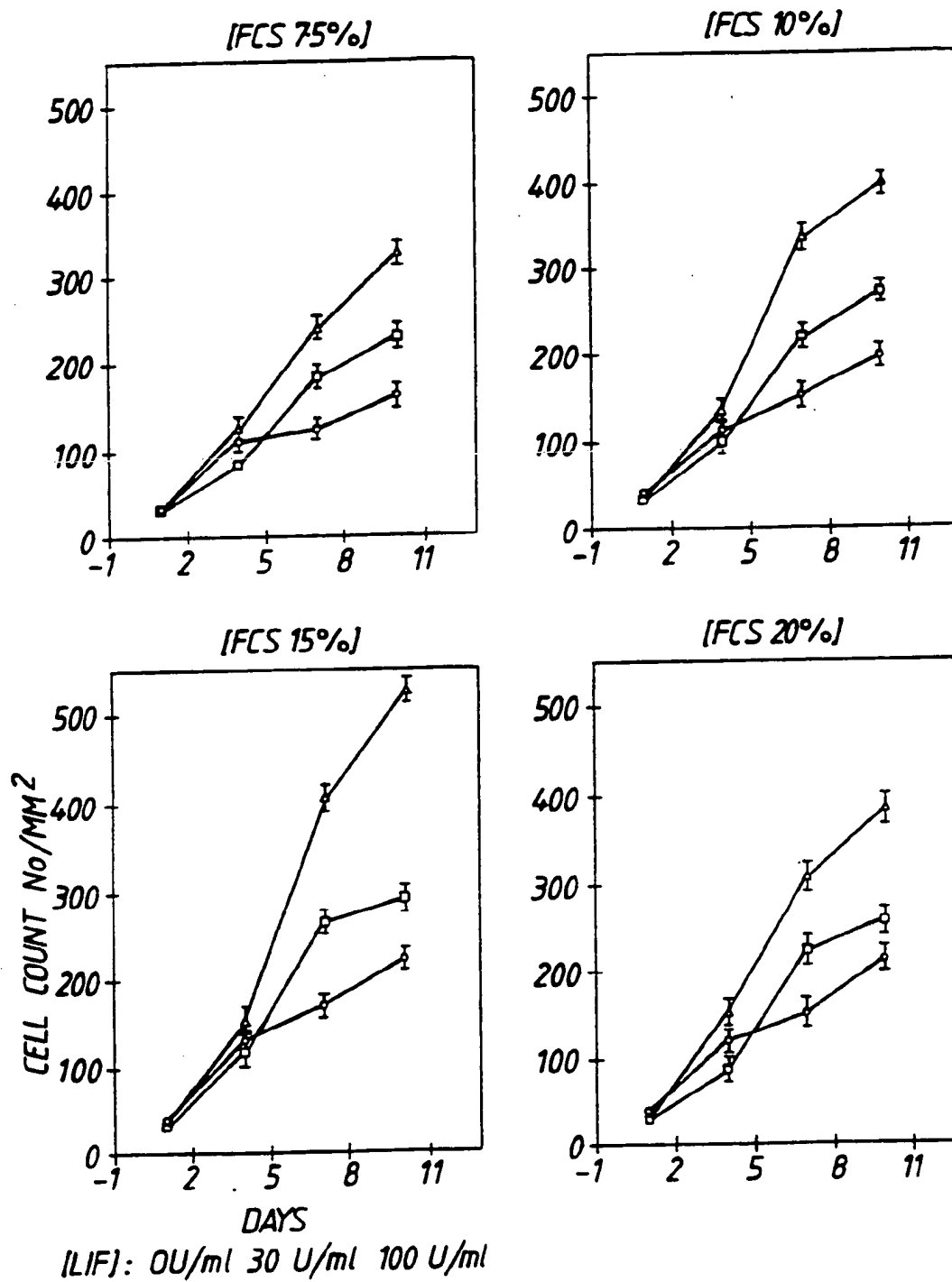
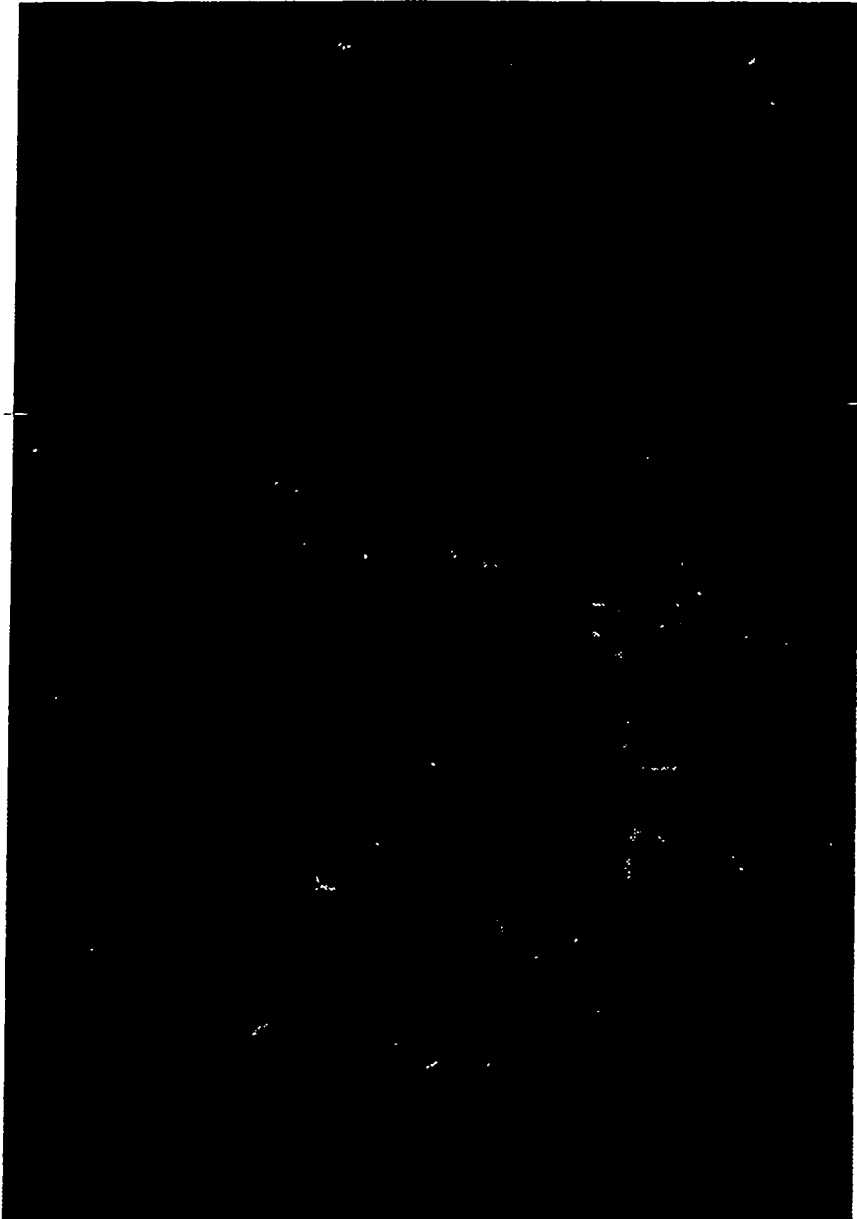


Fig.4B.

5/9



*Fig. 5a.*

SUBSTITUTE SHEET



6/9



*Fig. 5b.*

SUBSTITUTE SHEET

7/9



*Fig. 5c.*

SUBSTITUTE SHEET

8/9



*Fig. 6a.*

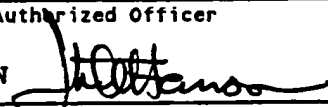
SUBSTITUTE SHEET

9/9



*Fig. 6b.*

SUBSTITUTE SHEET

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) 6				
According to International Patent Classification (IPC) or to both National Classification and IPC				
Int. Cl. <sup>5</sup> A61K 037/02, A61K 045/05				
<b>II. FIELDS SEARCHED</b>				
Minimum Documentation Searched 7				
Classification System	Classification Symbols			
IPC	A61K - Keywords: Leukaemia, Inhibit, Inhibitory Factor, Cholinergic, Neuronal Differentiation Factor C.A + B.A : As above			
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8				
MEDLINE				
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT</b> 9				
Category*	Citation of Document, with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13		
X,Y	AU,A, 15907/88 (AMRAD CORPORATION LIMITED) 6 October 1988 (06.10.88). See page 2 lines 14-19, page 3 lines 7-21, page 11 lines 5-22.	24-33		
P,X,Y	Journal of Immunology, Vol 143, No. 4, published 15 August 1989, H. Baumann and G.C. Wong, "Hepatocyte - Stimulating Factor III Shares Structural and Functional Identity with Leukaemia Inhibitory Factor.	24-33		
Y	Science, Vol. 246, published 15 December 1989, P.H. Patterson et al "Cholinergic Neuronal Differentiation Factor from Heart Cells is Identical to Leukaemia Inhibitory Factor". Pages 1412-1416.	24-33		
<p>* Special categories of cited documents: 10</p> <table style="width: 100%;"> <tr> <td style="width: 50%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p> </td> </tr> </table>			<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>
<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Z" document member of the same patent family</p>			
<b>IV. CERTIFICATION</b>				
Date of the Actual Completion of the International Search 26 February 1991 (26.02.91)	Date of Mailing of this International Search Report 1 March 1991			
International Searching Authority  Australian Patent Office	Signature of Authorized Officer  JOHN G. HANSON 			

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON  
INTERNATIONAL APPLICATION NO. PCT/AU 90/00556

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

---

Patent Document  
Cited in Search  
Report

Patent Family Members

---

AU 15907/88

EP 285448

WO 8807548

---

END OF ANNEX